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Molecular identification of *Azospirillum* spp.: Limitations of 16S rRNA and qualities of *rpo*D as genetic markers

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ABSTRACT

Since their discovery, plant-growth promoting rhizobacteria from the genus *Azospirillum* have been subjected to intensive research due to their biotechnological potential as crop inoculants. Phylogenetic analysis of *Azospirillum* spp. is carried out by 16S rRNA sequencing almost exclusively, but inconsistencies and low confidence often arise when working with close species. In this work, it was observed that these difficulties might be explained by a high number of rRNA operons with considerable intergenic variability within *Azospirillum* genomes. To search for alternative genetic markers from a list of housekeeping genes, the correlation between pairwise gene and whole-genome similarities was examined. Due to its good performance, *rpo*D was selected for further analyses. Genus-specific primers for the PCR-amplification and sequencing of *rpo*D from *Azospirillum* spp. were designed and tested on 16 type strains of different species. The sequences obtained were used for inferring a phylogenetic tree of the genus, which was in turn used as a reference to successfully identify a collection of 31 azospirilla isolated from many different locations of Argentine. In addition, several strains that might represent novel species were detected. The results indicate that the sequencing of *rpo*D is a suitable alternative method for a confident molecular identification in *Azospirillum* spp.

1. Introduction

Members of the genus *Azospirillum*, included in the *Rhodospirillaceae* family of Alphaproteobacteria, are free-living bacteria that inhabit soils of diverse ecological conditions, where they often associate to the rhizosphere or endosphere of plants. However, their metabolic versatility allows them to also live in harsh environments such as contaminated soils, road tar or sulfide springs. Plant-associated *Azospirillum* strains are of particular interest because they act like a probiotic by improving the health of their hosts. When applied as inoculants, they can promote plant growth by a diverse array of mechanisms, some of which are linked to production of plant growth regulators. Vast quantities of evidence gathered from decades of research prove the positive impact of azospirilla on the yield of extensive crops (Díaz-Zorita et al., 2015; Veresoglou and Menexes, 2010).

The taxonomic rank of *Azospirillum* as a genus was first proposed by Tarrand et al. (1978) after studying the morphology, physiology and DNA homology of a group of strains previously referred to as *Spirillum lipoferum*, and species *A. lipoferum* and *A. brasilense* were described. Since then, 15 more species were added to the genera: *A. amazonense* (Falk et al., 1985), *A. halopraeferens* (Reinhold et al., 1987), *A. irakense* (Khammas et al., 1989), *A. largimobile* (Ben Dekhil et al., 1997), *A. doebereinerae* (Eckert et al., 2001), *A. oryzae* (Xie and Yokota, 2005), *A. melinis* (Peng et al., 2006), *A. canadense* (Mehnaz et al., 2007a), *A. zeae* (Mehnaz et al., 2007b), *A. rugosum* (Young et al., 2008), *A. picis* (Lin et al., 2009), *A. thiophilum* (Lavrinenko et al., 2010), *A. formosense* (Lin et al., 2012), *A. humicireducens* (Zhou et al., 2013) and *A. fermentarium* (Lin et al., 2013). Four more species have been proposed but not officially accepted yet: *A. palatum* (Zhou et al., 2009), *A. himalayense* (Tyagi and Singh, 2014), *A. soli* (Lin et al., 2015) and *A. agricola* (Young et al., 2016). Later, *A. amazonense* and *A. irakense* were relocated to separate genera termed *Nitrospirillum* and *Niveispirillum*, respectively (Lin et al., 2014). Baldani and coworkers recently reviewed up-to-date information on the genus (Baldani et al., 2014).

The study of phylogenetic relationships between *Azospirillum* species is almost exclusively based on 16S rRNA sequences. Initial analyses that included the first 5 described species indicated that *A. brasilense*, *A. lipoferum* and *A. halopraeferens* forma solid lineage while *A. amazonense* and *A. irakense* segregate into an indepen-

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dent clade (Fani et al., 1995; Xia et al., 1994). With the addition of more species through the years, *A. brasilense* and *A. lipoferum* groups grew in complexity. As a consequence, low confidence is commonly obtained when defining phylogenetic inter-relationships between close species, particularly within *A. lipoferum* group, using 16S rRNA sequences. Inconsistencies have also been found when identifying new isolates (Vezyri et al., 2013).

Several limitations have been recognized when using 16S rRNA sequence in bacterial taxonomy. First, its slow evolution rate can lead to difficulties when resolving species that recently diverged or are too closely related (Fox et al., 1992). A second limitation is the fact that some genomes comprise multiple non-identical 16 rRNA genes. A survey carried out by Pei and co-workers using 883 prokaryotic genomes revealed up to 15 copies per genome with a mean intra-genomic diversity of 0.55% ± 1.46% (Pei et al., 2010). The presence of multiple 16S rRNA genes in *Azospirillum* genus was experimentally detected years ago (Caballero-Mellado et al., 1999) but their heterogeneity has not been studied. Additional difficulties associated with the use of 16S rRNA gene are the occurrence of homologous recombination (Teyssier et al., 2003) and horizontal gene transfer (HGT) events (Tian et al., 2015), both of particular concern in bacteria with dynamic genomes such as *Azospirillum*.

Whereas the commercial importance of *Azospirillum* motivates a compulsive recollection of new strains for agronomical exploitation, quick and reliable typing methods to identify new isolates, which would be of great value, are lacking. Here, we report the screening and selection of a new molecular marker for the genotyping of *Azospirillum* spp., and use it to analyse a collection of previously unidentified azospirilla.

2. Material and methods

2.1. Bacterial strains and growth conditions

Azospirillum type strains and isolates used in this work (Table 1) were routinely cultured at 30 °C for 48 h in nutrient broth (NB; Laboratorios Britania S.A., Buenos Aires, Argentine) and Congo Red medium (RC) (Rodriguez Caceres, 1982). Strain *A. rugosum* IMMIB AFH-6^T was obtained from Leibniz Institute DSMZ, Germany. Strains *A. humicireduncens* SgZ-5^T and *A. picis* IMMIB TAR-3^T were obtained from the Korean Agricultural Culture Collection (KACC) (Kim et al., 2009). Strains *A. oryzae* COC8^T, *A. lipoferum* Sp59b^T, *A. melinis* TMCY 0552^T and *A. palatum* ww10^T were kindly provided by Dr. Florence Wisniewski-Dyé from CNRS-Lyon University, France. Strains *A. zeae* N7^T and *A. canadense* DS2^T were kindly provided by Dr. Yuan Ze-Chun from Southern Crop Protection & Food Research Centre at Agriculture & Agri-Food Canada. Strain *A. doebereinerae* GSF-71^T was kindly provided by Dr. Marcela Montecchia from INBA-Buenos Aires University, Argentine. Strain *A. formosense* CC-Nfb-7^T was kindly provided by Dr. Fabricio Cassán from Río Cuarto National University, Argentine. Strains *A. fermentarium* CC-LY743^T, *A. soli* CC-LY788^T and *A. agricola* CC-HIH038^T were kindly provided by Dr. Chiu-Chung Young from National Chung Hsing University, Taiwan. *Azospirillum* sp. strains of the IMyZA-INTA collection were obtained from the PGPB Laboratory (IMyZA, INTA, Argentine) where they are routinely maintained.

2.2. Genome and gene sequence analyses

Complete genomic sequences of *Azospirillum* members (Table S1 in the online version at DOI: 10.1016/j.micres.2016.11.009) were retrieved from NCBI GenBank (http://www.ncbi.nlm.nih.gov/). Genome similarity indexes ANI and TETRA were calculated in JSpecies web server (http://jspecies.ribohost.com/jspeciesws/). ANI reciprocal values were averaged. GGDC indexes were computed by GGDC 2.0 server (http://ggdc.dsmz.de/distcalc2.php). Two-way AAI index was calculated using Kostas lab server (http://enve-omics.ce.gatech.edu/aai/). Nucleotide sequences of selected housekeeping genes (Table 2) were identified with BLAST (http://www.ncbi.nlm.nih.gov/blast), retrieved and used for multiple alignments with MAFFT available at the EMBL-EBI server (http://www.ebi.ac.uk/Tools/msa/mafft/). The resulting identity matrices were downloaded for further analyses. The correlation between pairwise nucleotide identity and genome similarity was analyzed by the non-parametric Spearman's rank correlation coefficient with Graphpad Prism 6 software (GraphPad Software Inc., California, USA).

2.3. Primer design and PCR conditions

To obtain DNA extracts, bacterial mass was recovered from a fresh streak in RC medium with a sterile loop, suspended in 50 μl of ultrapure water and boiled for 10 min. Extracts were stored at -20 °C until use.

Primer design for PCR-amplification of *rpo*D from *Azospirillum* spp. was based on a multiple alignment of available nucleotide sequences (Table 1). PCR reactions were performed with the T-Free DNA Polymerase kit (INBIO HIGHWAY®, Tandil, Argentine) in a final volume of 40 μl containing: 1 μl of DNA extract as template, 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 μM primer, 0.2 mM dNTP and 1 U of Taq Polymerase. When required, DMSO was added to a final concentration of 7.5%. The typical temperature cycling for *rpo*D amplification consisted of an initial 2 min denaturation step at 94 °C followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 64 °C for 45 s and extension at 72 °C for 1 min. PCR products were visualized by electrophoresis in 0.8% TAE-agarose gels stained with GelRed (Biotium, CA, USA). Bands were excised and purified using a silica suspension (Boyle and Lew, 1995). Purified PCR products were sequenced by the Genomics Department of INTA-Castelar, Argentine. GenBank accession codes for the sequences obtained in this work are detailed in Table 1.

2.4. Phylogenetic analyses

For phylogenetic analyses, all multiple alignments were performed with the MAFFT algorithm http://www.ebi.ac.uk/Tools/msa/. Tree inferences were carried out by the Maximum Likelihood (ML) method and Tamura-Nei substitution model implemented in MEGA 6 software (Tamura et al., 2013). Trees were tested by bootstrapping with 1000 iterations.

3. Results

3.1. Limitations of 16S rRNA gene in Azospirillum taxonomy

The suitability of 16S rRNA for the genotyping of *Azospirillum* sp. was examined. Nucleotide sequences of complete 16S rRNA genes present in *Azospirillum* genomes were detected using BLAST and retrieved for further analyses. Notably, nine to 10 different 16S rRNA genes were found within closed genomes, while only one to four were detected on incomplete or draft genomes (Fig. 1a and Table S1 in the online version at DOI: 10.1016/j.micres.2016.11.009). Multiple alignments showed a relatively high intra-genomic variability of 16S rRNA genes. Nucleotide identity of 100% was only observed in a few cases, while the lowest values obtained were 99.31, 99.1, 99.31, 98.27 and 99.24% for strains Az39, Sp7^T, BV-S^T and 4B and B510, respectively (Fig. 1a). Strain 4B had the highest inter-genic divergence, showing a mean diversity of 0.94 ± 0.14%. The lower values for this strain were even below pairwise identities obtained between some *Azospirillum* type strains; e.g. *A. lipoferum* Sp59b^T and *A. melinis* TMCY0552^T shared

Table 1
Strains and sequences analyzed.

Strain	GenBank nucleotide accession code		
	16S rRNA	*nif*H	*rpo*D
Azospirillum strains			
A. lipoferum Sp59b^T	GU256441	DQ787334	KX774701[c]
A. brasilense Sp7^T	GU256438	AMK58_04660[a]	AMK58_09830[a]
A. halopraeferens Au4^T	GU256439	AUCF01000008[b]	AUCF01000018[b]
A. oryzae COC8^T	GU256443	AB185395	KX774696[c]
A. largimobile UQM 2041^T	X90759	–	–
A. doebereinerae GSF-71^T	AJ238567	FJ799358	KX774704[c]
A. melinis TMCY 0552^T	GU256442	GU256450	KX774700[c]
A. zeae N7^T	DQ682470	DQ682472	KX774702[c]
A. canadense DS2^T	HM636056	GU256446	KX774703[c]
A. rugosum IMMIB AFH-6^T	AM419042	GU256452	KX774692[c]
A. palatum ww10^T	EU747318	–	KX774697[c]
A. picis IMMIB TAR-3^T	AM922283	GU256451	KX774699[c]
A. thiophilum BV-S^T	AL072_05050[a]	AL072_00480[a]	AL072_09785[a]
A. formosense CC-Nfb-7^T	GU256444	HM193519	KX774698[c]
A. fermentarium CC-Ly743^T	JX843282	JX857866	KX774693[c]
A. humicireducens SgZ-5^T	JX274435	JX294516	A6A40_03765[a]
A. himalayense plt-3^T	GQ284588	GQ249665	JF910091
A. soli CC-Ly788^T	KC297124	KC297125	KX774694[c]
A. agricola CC-HIH038^T	KR296799	KT634300	KX774695[c]
A. lipoferum 4B	–	–	AZOLI_0932[a]
Azospirillum sp. B510	–	–	AZL_010580[a]
A. brasilense Sp245	–	–	AZOBR_150128[a]
A. brasilense Cd	–	–	KX774736[c]
A. brasilense Az39	–	–	ABAZ39_04200[a]
Azospirillum sp. Az1	–	–	KX774724[c]
Azospirillum sp. Az3	–	–	KX774706[c]
Azospirillum sp. Az4	–	–	KX774725[c]
Azospirillum sp. Az5	–	–	KX774726[c]
Azospirillum sp. Az6	–	–	KX774710[c]
Azospirillum sp. Az8	–	–	KX774707[c]
Azospirillum sp. Az9	–	–	KX774711[c]
Azospirillum sp. Az10	–	–	KX774727[c]
Azospirillum sp. Az11	–	–	KX774728[c]
Azospirillum sp. Az17	–	–	KX774729[c]
Azospirillum sp. Az18	–	–	KX774712[c]
Azospirillum sp. Az19	–	–	KX774708[c]
Azospirillum sp. Az23	–	–	KX774713[c]
Azospirillum sp. Az24	–	–	KX774730[c]
Azospirillum sp. Az32	–	–	KX774731[c]
Azospirillum sp. Az35	–	–	KX774732[c]
Azospirillum sp. Az36	–	–	KX774733[c]
Azospirillum sp. Az37	–	–	KX774734[c]
Azospirillum sp. Az40	–	–	KX774714[c]
Azospirillum sp. Az45	–	–	KX774735[c]
Azospirillum sp. Az48	–	–	KX774715[c]
Azospirillum sp. Az52	–	–	KX774716[c]
Azospirillum sp. Az55	–	–	KX774717[c]
Azospirillum sp. Az60	–	–	KX774718[c]
Azospirillum sp. Az63	–	–	KX774709[c]
Azospirillum sp. Az65	–	–	KX774719[c]
Azospirillum sp. Az66	–	–	KX774720[c]
Azospirillum sp. Az71	–	–	KX774705[c]
Azospirillum sp. Az72	–	–	KX774721[c]
Azospirillum sp. Az73	–	–	KX774722[c]
Azospirillum sp. Az74	–	–	KX774723[c]
Other strains			
S. stibiiresistens SB22^T	NR_117972	N825_07105[a]	N825_10320[a]
S. aerolata 5416T-32^T	NR_043929	N826_14335[a]	N826_05105[a]
N. amazonense Y2	GU256437	AZA_90011[a]	AZA_18308[a]
N. irakense KBC1^T	GU256440	AUCG01000004[b]	AUCG01000014[b]
R. centenaria SW^T	RC1_2630[a]	RC1_3683[a]	RC1_2006[a]
R. rubrum S1^T	Rru_AR0004[a]	Rru_A1010[a]	Rru_A2882[a]
B. japonicum E109	RN69_07475[a]	RN69_39105[a]	RN69_09645[a]

[a] Genomic locus tag.
[b] Locus not annotated in draft genome.
[c] Sequences obtained in this work.

a 16S rRNA nucleotide identity of 98.89% (Fig. S1 in the online version at DOI: 10.1016/j.micres.2016.11.009). As expected, phylogenetic analyses using the different 16S rRNA genes of strain 4B led to ambiguous results. For example, genes 16S rRNA chr-1 and p1-3 grouped strain 4B with *A. humicireducens* SgZ-5^T, while 16S rRNA p3-1 and p5-1 indicated that 4B is more related to *A. zeae* N7^T and *A. oryzae* COC8^T (Fig. 1b). Similarly, compgen tool implemented in the EzTaxon server (http://www.ezbiocloud.net/

Table 2
Results on the analyzed housekeeping genes.

Gene	Copies	Genomic location	Locus[a]	Spearman correlation (r^2)					
				ANIb	ANIm	GGDCb	GGDCm	AAI	TETRA
*atp*D	1	Chromosome	AZOBR_RS04010	0,936	0,927	0,936	0,907	0,897	0,777
*cpn*60	2	Chromosome/p1	AZOBR_RS02500/AZOBR_RS17855	0,888	0,970*	0,943	0,897	0,882	0,726
*dna*K	2[b]	Chromosome/p1	AZOBR_RS02240/AZOBR_RS17850	0,914	0,887	0,872	0,915	0,894	0,783
*glm*S	1	Chromosome/p1[c]	AZOBR_RS09675	0,576	0,579	0,534	0,583	0,556	0,518
*gln*A	1	Chromosome	AZOBR_RS07095	0,851	0,963	0,934	0,853	0,849	0,743
*gps*A	1	Chromosome	AZOBR_RS00325	0,812	0,825	0,767	0,783	0,778	0,607
*gyr*B	1	Chromosome/p1[d]	AZOBR_RS17675	0,865	0,928	0,912	0,869	0,850	0,717
hisC1	1	Chromosome	AZOBR_RS08675	0,915	0,857	0,902	0,894	0,884	0,722
*lig*A	1	Chromosome	AZOBR_RS09960	0,915	0,947	0,951*	0,896	0,891	0,722
*mut*L	1	Chromosome	AZOBR_RS05025	0,872	0,935	0,940	0,860	0,855	0,723
*nif*H	1	Chromosome	AZOBR_RS05580	0,885	0,819	0,872	0,843	0,851	0,893*
*pan*C	1	Chromosome/p1[c]	AZOBR_RS09715	0,893	0,877	0,857	0,924	0,912	0,779
*phe*S	1	Chromosome	AZOBR_RS00895	0,852	0,929	0,913	0,872	0,850	0,692
*rec*A	1	Chromosome	AZOBR_RS11095	0,960*	0,872	0,926	0,937	0,921	0,776
*rpo*D	1	Chromosome	AZOBR_RS10905	0,955*	0,981*	0,947*	0,968*	0,961*	0,833*
*trp*E	1	Chromosome	AZOBR_RS07490	0,920	0,884	0,887	0,942*	0,945*	0,801

[a] Loci codes of *A. brasilense* Sp245 genome in GenBank datsabase.
[b] Second copy present in replicon p1 of *A. brasilense* strains only.
[c] Gene coded in replicon p1 of strains 4B and B510.
[d] Gene coded in replicon p1 of *A. brasilense* strains.
* Top-two highest correlation values.

eztaxon/compgen) identified strain 4B as more similar to *A. thiophilum*, *A. oryzae* or *A. lipoferum* species depending on which 16S rRNA gene was used as the query (Table S2 in the online version at DOI: 10.1016/j.micres.2016.11.009).

3.2. *Screening genetic markers for* Azospirillum *genotyping*

Pairwise genome similarity of *Azospirillum* genomes was calculated by different indexes: GGDC, ANI, TETRA and AAI. The sequence of *A. humicireducens* SgZ-5^T main chromosome was recently uploaded to GenBank, but since no data is available for other expected replicons it was excluded from the analyses to prevent biases. It is important to note that the genome similarity indexes showed discrepancies on the taxonomy of strains Sp7^T, Sp245 and Az39, according to the established threshold values for species delimitation in bacteria (Auch et al., 2010; Konstantinidis and Tiedje, 2005; Richter et al., 2015). BLAST-based indexes (ANIb and GGDCb) indicated that the these strains belong to three different species, while MUMmer-based ones (ANIm and GGDCm), which perform better on closer genomes (Richter et al., 2015), suggested that Az39 and Sp245 belong to the same species but different from Sp7^T. On the other hand, according to index AAI the three strains were part of the same species (Fig. S2 in the online version at DOI: 10.1016/j.micres.2016.11.009).

The calculated genome similarity indexes were used as a guide for screening potential *Azospirillum* genetic markers from a list of housekeeping genes commonly used for MLSA in other genera. The analysis of *Azospirillum* genomes indicated that most of the genes are chromosomally encoded, although in certain strains some of them are located in replicon p1. In addition, two copies of the genes *cpn*60 and *dna*K were detected in different locations of the genomes (Table 2). Nucleotide sequences were retrieved and correlation between pairwise gene nucleotide identity and genome similarity was determined. Overall, *rpo*D showed the best correlation as evidenced by its top-two position in the different rankings, while *glm*S had the worst correlation in all of them. Performance of the other genes depended on the genomic index that was used for calculations (Table 2). For example, *lig*A gene variability was well correlated to GGDCb index, but showed moderate performance for the rest of the indexes. A plot of pairwise *rpo*D nucleotide identity vs. genome similarity showed a non-linear correlation between both data sets. Species boundary according to GGDC (70%) and ANI (95%) corresponded approximately to an *rpo*D nucleotide identity of 98.9-99% (Fig. S3 in the online version at DOI: 10.1016/j.micres.2016.11.009).

3.3. *Phylogenetic analysis of* Azospirillum *genera by rpoD sequencing*

Next, a PCR assay for the partial amplification and sequencing of *rpo*D was developed. Available *rpo*D sequences from *Azospirillum* (Table 1) and *Nitrospirillum* - former members of *Azospirillum* genera- strains were subjected to multiple alignment and primers were designed in regions of high nucleotide conservation. One forward primer termed Azo.rpoD F (5′-GAGATGGGCATCAACATCGT-3′) and two reverse primers named Azo.rpoD R (5′-CCTTCATCAGGCCGATGTTGCC-3′) and Azo.rpoD R2 (5′-ACCTTCTCCAGCGGCATCATC-3′) were designed and combined to successfully amplify fragments of around 870 and 1100 bp, respectively, from genomic DNA of different *Azospirillum* species (Fig. 2a). Amplification of *rpo*D from some of the strains required the addition of DMSO to the reaction (data not shown). Thus, primers Azo.rpoD F/Azo.rpoD R2 and DMSO were typically used for subsequent determinations.

The developed PCR assay was used to obtain partial sequences of *rpo*D from all of the *Azospirillum* type strains described up to date, with the exception of *A. largimobile*, whose type strain is unavailable (Yarza et al., 2013), and *A. himalayense,* that already has a partial *rpo*D sequence available at GenBank (Table 1). These sequences were used to infer a ML phylogenetic tree that was overall well supported by bootstrap testing and depicted *Azospirillum* genus as a solid lineage (Fig. 2b). Species *A. fermentarium, A. halopraeferens* and *A. palatum* diverged into distinct individual lineages. The rest of the species grouped under a common ancestor and divided into two complex sub-groups, that will be here termed clade B (for *brasilense*) and clade L (for *lipoferum*) (Fig. 2b). Clade B was composed by species *A. brasilense*, *A. formosense*, *A. himalayense*, *A. doebereinerae*, *A. canadense, A. rugosum* and, more distantly, *A. soli.* Intra-group pairwise nucleotide identity ranged from a minimum of 91.63 between strains *A. himalayense* plt-3^T and *A. soli* CC-Ly788^T, to a maximum of 98.86 between strains *A. canadense* DS2^T and *A. rugosum* IMMIB-AFH6^T (Fig. S4 in the online version at DOI: 10.1016/j.micres.2016.11.009), which is just below the approximate *rpo*D identity value that correspond to the species threshold accord-

a

Sp7	Chr rRNA1	Chr rRNA2	p1 rRNA1	p1 rRNA2	p1 rRNA3	p2 rRNA1	p2 rRNA2	p3 rRNA1	p3 rRNA2	p3 rRNA3
Chr rRNA1	100	99.24	100	99.93	99.24	99.17	99.17	100	99.17	99.17
Chr rRNA2		100	99.24	99.17	100	99.93	99.93	99.24	99.93	99.93
p1 rRNA1			100	99.93	99.24	99.17	99.17	100	99.17	99.17
p1 rRNA2				100	99.17	99.1	99.1	99.93	99.1	99.1
p1 rRNA3					100	99.93	99.93	99.24	99.93	99.93
p2 rRNA1						100	100	99.17	100	100
p2 rRNA2							100	99.17	100	100
p3 rRNA1								100	99.17	99.17
p3 rRNA2									100	100
p3 rRNA3										100

Az39	Chr rRNA1	Chr rRNA2	p1 rRNA1	p1 rRNA2	p1 rRNA3	p1 rRNA4	p2 rRNA1	p4 rRNA1	p4 rRNA2
Chr rRNA1	100	99.45	99.72	99.72	99.52	99.45	99.65	99.45	99.31
Chr rRNA2		100	99.72	99.72	99.93	100	99.79	99.52	99.86
p1 rRNA1			100	100	99.79	99.72	99.93	99.72	99.59
p1 rRNA2				100	99.79	99.72	99.93	99.72	99.59
p1 rRNA3					100	99.93	99.72	99.52	99.79
p1 rRNA4						100	99.79	99.52	99.86
p2 rRNA1							100	99.72	99.65
p4 rRNA1								100	99.38
p4 rRNA2									100

BV-S	Chr rRNA1	Chr rRNA2	p1 rRNA1	p1 rRNA2	p2 rRNA1	p2 rRNA2	p4 rRNA1	p4 rRNA2	p5 rRNA1
Chr rRNA1	100	100	99.45	99.93	99.79	99.86	99.52	99.86	99.52
Chr rRNA2		100	99.45	99.93	99.79	99.86	99.52	99.86	99.52
p1 rRNA1			100	99.52	99.38	99.31	99.93	99.59	99.93
p1 rRNA2				100	99.72	99.79	99.45	99.93	99.59
p2 rRNA1					100	99.93	99.45	99.79	99.45
p2 rRNA2						100	99.38	99.72	99.38
p4 rRNA1							100	99.52	99.86
p4 rRNA2								100	99.65
p5 rRNA1									100

4B	Chr rRNA1	Chr rRNA2	p1 rRNA1	p1 rRNA2	p1 rRNA3	p2 rRNA1	p2 rRNA2	p3 rRNA1	p5 rRNA1
Chr rRNA1	100	98.76	99.03	99.45	99.38	99.65	99.52	98.96	98.96
Chr rRNA2		100	98.76	98.76	98.27	98.96	99.1	98.27	98.27
p1 rRNA1			100	99.59	98.69	99.38	99.24	98.69	98.69
p1 rRNA2				100	99.1	99.38	99.24	98.69	98.69
p1 rRNA3					100	99.31	99.17	98.62	98.62
p2 rRNA1						100	99.86	99.31	99.31
p2 rRNA2							100	99.17	99.17
p3 rRNA1								100	100
p5 rRNA1									100

B510	Chr rRNA1	Chr rRNA2	p1 rRNA1	p1 rRNA2	p2 rRNA1	p2 rRNA2	p4 rRNA1	p4 rRNA2	p5 rRNA1
Chr rRNA1	100	99.31	100	100	100	99.38	99.24	99.24	100
Chr rRNA2		100	99.31	99.31	99.31	99.93	99.65	99.65	99.31
p1 rRNA1			100	100	100	99.38	99.24	99.24	100
p1 rRNA2				100	100	99.38	99.24	99.24	100
p1 rRNA3					100	99.38	99.24	99.24	100
p1 rRNA4						100	99.72	99.72	99.38
p2 rRNA1							100	100	99.24
p4 rRNA2								100	99.24
p5 rRNA1									100

b

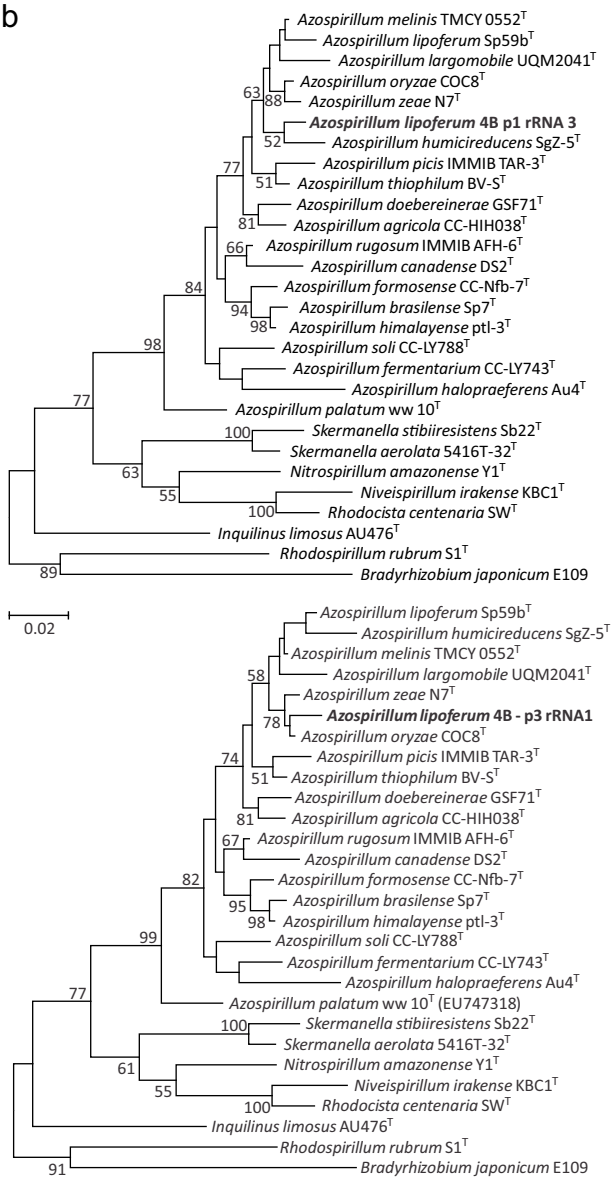

Fig. 1. Intra-genomic variability of 16S rRNA genes present in *Azospirillum* genomes. a) Multiple 16S rRNA genes present in the genome of *A. brasilense* Sp7^T and Az39, *A. thiophilum* BV-S^T, *A. lipoferum* 4B and *Azospirillum* sp. B510, were retrieved from Genbank. The loci codes of the different 16S rRNA genes used in the analysis are *A. brasilense* Sp7^T Chr rRNA1 (AMK58_RS13100), Chr rRNA2 (AMK58_RS08015), p1 rRNA1 (AMK58_RS20620), p1 rRNA2 (AMK58_RS21495), p1 rRNA3 (AMK58_RS18720), p2 rRNA1 (AMK58_23395), p2 rRNA2 (AMK58_22235), p3 rRNA1 (AMK58_25190), p3 rRNA2 (AMK58_26725), p3 rRNA3 (AMK58_27540); *A. brasilense* Az39 Chr rRNA1 (ABAZ39_06935), Chr rRNA2 (ABAZ39_13275), p1 rRNA1 (ABAZ39_15090), p1 rRNA2 (ABAZ39_15930), p1 rRNA3 (ABAZ39_21670), p1 rRNA4 (ABAZ39_20730), p2 rRNA1 (ABAZ39_26570), p4 rRNA1 (ABAZ39_32260), p4 rRNA2 (ABAZ39_31750); *A. thiophilum* BV-S^T Chr rRNA1 (AL072_06320), Chr rRNA2 (AL072_05050), p1 rRNA1 (AL072_15845), p1 rRNA2 (AL072_14160), p2 rRNA1 (AL072_22640), p2 rRNA2 (AL072_19465), p4 rRNA1 (AL072_28085), p4 rRNA2 (AL072_26515), p5 rRNA1 (AL072_31315); *A. lipoferum* 4B Chr rRNA1 (AZOLI_16s_rRNA_2), Chr rRNA2 (AZOLI_16s_rRNA_1), p1 rRNA1 (AZOLI_p1_16s_rRNA_1), p1 rRNA2 (AZOLI_p1_16s_rRNA_3), p1 rRNA3 (AZOLI_p1_16s_rRNA_2), p2 rRNA1 (AZOLI_p2_16s_rRNA_1), p2 rRNA2 (AZOLI_p2_16s_rRNA_2), p3 rRNA1 (AZOLI_p3_16s_rRNA_1), p5 rRNA1 (AZOLI_p5_16s_rRNA_1); and *Azospirillum* sp. B510 Chr rRNA1 (AZL_r007), Chr rRNA2 (AZL_r053), p1 rRNA1 (AZL_ra07), p1 rRNA2 (AZL_ra13), p1 rRNA3 (AZL_ra19), p1 rRNA4 (AZL_ra25), p2 rRNA1 (AZL_rb05), p3 rRNA1 (AZL_rc06), p5 rRNA1 (AZL_re01). The sequences were trimmed to positions 27–1492 and subjected to multiple alignments. The resulting nucleotide identity matrixes are shown. The lowest value of each matrix is highlighted in a grey box. b) Two different 16S rRNA genes of strain 4B were individually used for phylogenetic analyses by multiple alignment with sequences of *Azospirillum* type strains and tree inference by the Maximum Likelihood method, using the Tamura-Nei substitution model and a Bootstrap testing of 1000 iterations. Other members of the *Rhodospirillaceae* family and *Bradyrhizobium japonicum* E109 were used as out-group. Genbank accession codes of the sequences used for the analyses are detailed in Table 1. Strain 4B is highlighted in bold.

ing to the calibration with genome similarity (Fig. S3 in the online version at DOI: 10.1016/j.micres.2016.11.009). Clade L included species *A. lipoferum*, *A. melinis*, *A. humicireducens*, *A. zeae*, *A. oryzae*, *A. picis*, *A. thiophilum* and *A. agricola.* This clade was more variable, with identity values spanning from 90.49 for *A. melinis* TMCY 0552^T-*A. agricola* CC-HIH038^T to 95.98 for *A. lipoferum* Sp59b^T-*A. oryzae* COC8^T pairs, respectively (Fig. S4 in the online version at DOI: 10.1016/j.micres.2016.11.009). The tree structure was similar to an equivalent 16S rRNA-based ML tree (Fig. 3a), but differing in the position of certain species within the clades B and L. The clearest example is strain *A. doebereinerae* GSF-71^T that was well-supported into clade B by *rpo*D but belonged to clade L, close to *A. agricola* species, according to 16S rRNA (Fig. 3a). Similarly, ML trees inferred with the concatenated alignments of 16S rRNA+*rpo*D, *rpo*D+*nif*H and 16S rRNA+*rpo*D+*nif*H also supported the existence of clades B and L, but did not coincide with the *rpo*D tree in the inner

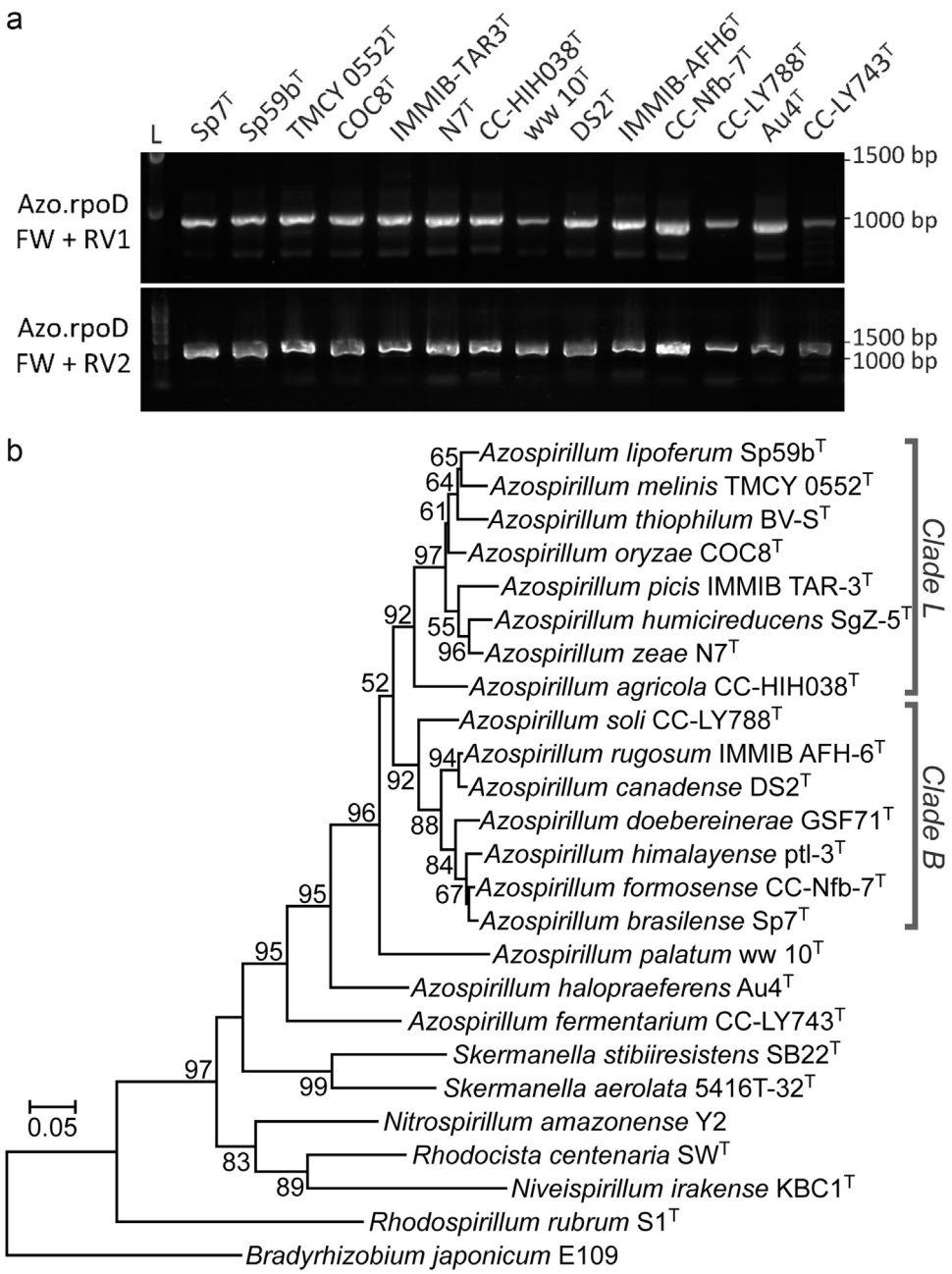

Fig. 2. PCR amplification and phylogenetic reconstruction of *Azospirillum* genera using *rpo*D gene sequence. a) Two pairs of primers for the genus-specific PCR amplification and sequencing of *rpo*D fragments of ~870 (upper panel) and 1100 bp (lower panel) were designed and tested on several *Azospirillum* type strains. Amplification products were separated by electrophoresis in a 0.8% agarose gel. The strain used for amplification is indicated above each line. A 1 kb molecular marker was included (L). **b)** PCR amplification and sequencing of *rpo*D was performed on 13 *Azospirillum* type strains. The resulting sequences were aligned and phylogenetic reconstruction of the genus by the Maximum Likelihood method, using the Tamura-Nei substitution model and a Bootstrap testing of 1000 iterations. Bootstrap values ≥50 are shown in the corresponding nodes. Other members of the *Rhodospirillaceae* family and *Bradyrhizobium japonicum* E109 were used as out-group. Genbank accession codes of the obtained sequences are detailed in Table 1.

distribution of species within them. The inclusion of 16S rRNA in the concatenation led to low confidence in clade L branches, while the ML tree based on *rpo*D+*nif*H alignment, which excludes the 16S rRNA data, showed higher bootstrap support (Fig. S5 in the online version at DOI: 10.1016/j.micres.2016.11.009).

3.4. *Genotyping* Azospirillum *isolates by rpoD sequencing*

To demonstrate the applicability of the developed assay, 31 *Azospirillum* isolates that are maintained in the Culture Collection of Plant Growth-Promoting Bacteria Laboratory of IMyZA-INTA, Argentina, was typified by the amplification and sequencing of *rpo*D. The resulting sequences were used for phylogenetic analysis by multiple alignment and inference of a ML tree using *Azospirillum* type strains as a reference. The model strains of sequenced genome (Table S1 in the online version at DOI: 10.1016/j.micres.2016.11.009) and the broadly studied strain Cd (Eskew et al., 1977) were also included in the analysis.

As shown in Fig. 3, all of the isolates were included in clade B. Most of them were positioned close to *A. brasilense* strains Sp7T, Sp245 and Az39, *A. formosense* CC-Nfb-7T and *A. himalayense* plt-3T. Several distant clades that were well supported by bootstrap

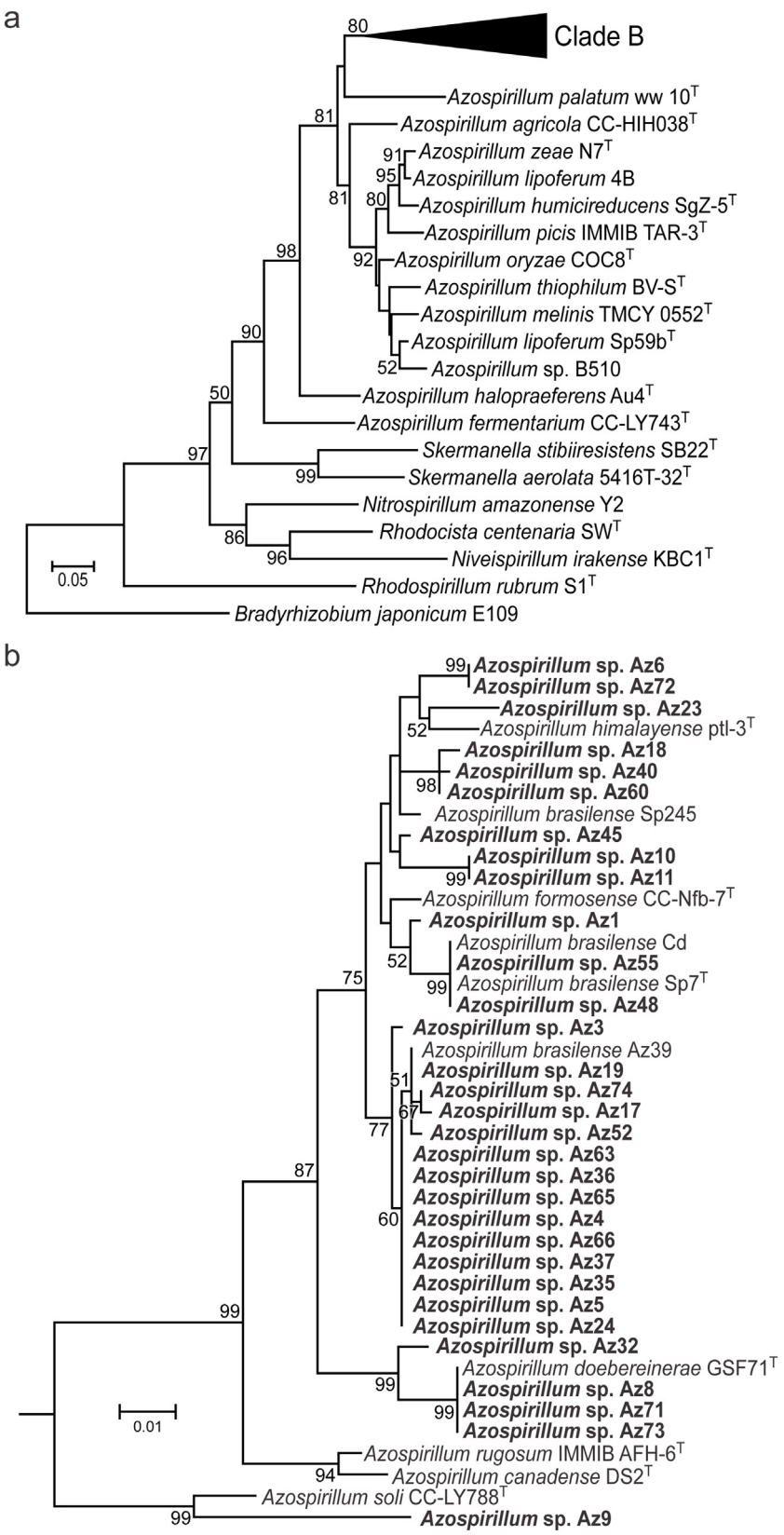

Fig. 3. Molecular genotyping of an *Azospirillum* sp. collection by *rpo*D sequencing. A collection of 31 *Azospirillum* sp. strains (bold) were genotyped by *rpo*D-sequencing. The obtained sequences were aligned along with type and reference strains, and a phylogenetic tree was inferred by the Maximum Likelihood method, Tamura-Nei substitution model and a Bootstrap testing of 1000 iterations. Bootstrap values ≥50 are shown in the corresponding nodes. Other members of the *Rhodospirillaceae* family and *Bradyrhizobium japonicum* E109 were used as outgroups. Genbank accession codes of the sequences are detailed in Table 1. A condensed tree (a) and a close up of clade B (b) are shown.

Table 3
Phylogenetic grouping of *Azospirillum* sp. strains according to *rpo*D sequence.

Strain	Closest species	Identity to type strain	Allocated species	Intra-group identity
Az39	*A. brasilense*	98.75	*A. brasilense*	98.25–100
Sp245	*A. brasilense*	98.75		
Az1	*A. brasilense*	98.87		
Az3	*A. brasilense*	99.02		
Az4	*A. brasilense*	98.93		
Az5	*A. brasilense*	98.92		
Az19	*A. brasilense*	98.85		
Az24	*A. brasilense*	98.98		
Az35	*A. brasilense*	98.93		
Az36	*A. brasilense*	98.92		
Az37	*A. brasilense*	98.93		
Az45	*A. brasilense*	98.73		
Az48	*A. brasilense*	100		
Az52	*A. brasilense*	98.96		
Az55	*A. brasilense*	100		
Az63	*A. brasilense*	98.79		
Az65	*A. brasilense*	99.01		
Az66	*A. brasilense*	98.93		
Az8	*A. doebereinerae*	100	*A. doebereinerae*	100
Az71	*A. doebereinerae*	100		
Az73	*A. doebereinerae*	100		
Az6	*A. himalayense*	98.00	Undescribed sp. 1	100
Az72	*A. himalayense*	98.00		
Az32	*A. doebereinerae*	98.21	Undescribed sp. 2	–
Az9	*A. soli*	96.36	Undescribed sp. 3	–
Az10	*A. formosense*	98.19	Undescribed sp. 4	100
Az11	*A. formosense*	98.21		
Az17	*A. brasilense*	98,45	Undescribed sp. 5	99.9
Az74	*A. brasilense*	98,49		
Az18	*A. formosense*	98.09	Undescribed sp. 6	99.2–99.8
Az40	*A. brasilense*	97.96		
Az60	*A. formosense*	98.31		
Az23	*A. himalayense*	98.01	Undescribed sp. 7	–

and shared low nucleotide identity with reference strains were observed (Table 3). The *A. doebereinerae* branch included strains GSF-71^T, Az8, Az71 and Az73 sharing 100% *rpo*D nucleotide identity, and also Az32 in a more distant position (98.21% identity to GSF-71^T). Isolate Az9 and *A. soli* LY788^T formed a single clade, sharing 96.36% identity (Table 3). As expected, *A. formosense* CC-Nfb-7^T shared high nucleotide identity with *A. brasilense* strains Sp7^T, Az39 and Sp245 (98,3–98,53%), and strain Cd showed 100% identity to its parental strain Sp7^T (Eskew et al., 1977) (Fig. S6 in the online version at DOI: 10.1016/j.micres.2016.11.009). In addition, model strains *A. lipoferum* 4B and *Azospirillum* sp. B510 were positioned in clade L close to *A. zeae* N7^T (97.06% identity) and *A. lipoferum* Sp59b^T (95.43% identity), respectively (Fig. 3a).

4. Discussion

In prokaryotic taxonomy, the 16S rRNA gene is used as a standard genetic marker for the identification and phylogenetic study of new strains. However, difficulties associated with the use of this gene are well documented (Janda and Abbott, 2007). In this work, genomic analyses evidenced the presence of multiple and heterogeneous rRNA operons (*rrn*) within individual genomes of *Azospirillum* strains. Intra-genomic heterogeneity of 16S rRNA genes was higher in *A. lipoferum* 4B and led to ambiguities while trying to detect its closest relatives within the genus. Thus, our results advice against the use of 16S rRNA nucleotide identity as a single taxonomic guide for comparing closely related *Azospirillum* species.

It is generally believed that, due to structural and functional constraints, concerted evolution acts in bacteria that bear multiple *rrn*, homogenizing them and maintaining variability below a certain limit (Hillis et al., 1991). However, our results call into question the prevalence of this mechanism in *Azospirillum* genus, or at least in some strains. The high heterogeneity of *rrn* evidenced here rather suggests that HGT strongly shapes *Azospirillum* genomes, partially overriding concerted evolution. Genomic plasticity that would explain this scenario is not unexpected in the genus (Vial et al., 2006). Similar observations have been made in other genera such as *Aeromonas*, which is a paradigmatic case of phylogenetic incongruence due to intra-genomic heterogeneity of 16S rRNA genes. Homologous recombination and HGT were also pointed out as the mechanisms responsible of *rrn* heterogeneity (Sneath, 1993).

The fact that taxonomic difficulties in *Azospirillum* are due, in part, to genome complexity is illustrated by genomic comparisons: strain relatedness was highly dependent on the algorithm used to measure genome similarity. DNA-based indexes split *A. brasilense* strains into two or three species when alignments were performed with MUMmer or BLAST, respectively. Index AAI, which relies on protein data and is thus less prone to be affected by genomic dynamics, grouped the three strains in the same species. It is evident that much work will be required to bring *Azospirillum* phylogeny to a deeper understanding.

In the search for alternative genetic markers, some authors have turned their attention to the 16S-23S rRNA internal transcribed spacer for a source of inter-species genetic variability in *Azospirillum* (Jijón-Moreno et al., 2015; Vezyri et al., 2013). However, it may suffer from the same limitations than 16S rRNA (i.e. multiple heterogeneous copies). An additional gene commonly used in *Azospirillum* taxonomy is *nif*H, although it displays two problems: a) the amplified PCR fragment is too short to produce phylogenetic trees of good confidence, and b) it is not suitable for non-diazotrophic azospirilla such as *A. palatum* ww10^T. The *cpn*60 gene that has been occasionally used in *Azospirillum* (Mehnaz et al., 2007a, 2007b) is duplicated and would generate confusing results; this seems to be the case of *cpn*60 sequences published for *A. zeae* N7^T and *A. oryzae* COC8^T (results not shown). A third

gene used for the genotyping *Azospirillum* sp. is *dnaK* (Vezyri et al., 2013), but it also displays a duplication in the replicon p1 of *A. brasilense* strains. Taken all the facts together, it is evident that new and suitable genetic markers are needed for taxonomic studies in *Azospirillum*. Recently, Jijón-Moreno and co-workers carried out a diversity survey of several *A. brasilense* and *A. lipoferum* strains using the auxin-related genes *ipdC*, *hisC1* and *hisC2*. They found that *hisC* genes allowed the differentiation of both species, but the study was not extended to other species (Jijón-Moreno et al., 2015). In this work, using gene-genome similarity correlation as a selection criterion, the *rpoD* gene was detected as a candidate molecular marker suitable for genotyping of *Azospirillum* spp. The analysis showed that it is a single-copy gene encoded in the main chromosome and that it shows good correlation to genome similarity. The suitability of this gene for taxonomic studies in bacteria is not surprising, since it has already been adopted in other genera such as *Pseudomonas* (Mulet et al., 2009), *Frankia* (Bernèche-D'Amours et al., 2011) and – notably – in *Aeromonas* (Soler et al., 2004). The available genomic data allowed us to design genus-specific *rpoD* primers that were successfully tested on *Azospirillum* spp. and used for molecular typing of unidentified azospirilla. Moreover, the use of these primers may also be extended to other related genera such as *Nitrosprillum* or *Niveispirillum*.

A basic step towards the use of *rpoD* for the genotyping of azospirilla was the disposal of reference sequences within the genus. Reconstruction of *Azospirillum* phylogeny by *rpoD*-sequencing yielded a robust phylogenetic tree with improved confidence over the 16S rRNA-based tree. Two complex clades, here termed clade L and clade B,were detected in equivalence to the initial separation of the genus into species *A. lipoferum* and *A. brasilense* by Tarrand et al. (1978). These clades could also be detected in phylogenetic trees that were based on the 16S rRNA gene or on concatenated alignments, but with differences on the position of some species. It must be noted that, even when it was strongly supported by the genetic markers, the grouping of species into these clades had no basis on morphological, physiological or biochemical characteristics (Baldani et al., 2014). We speculate that this discrepancy is due to intense inter-species HGT, which will be prone to confirmation when new genomic data of *Azospirillum* species becomes available.

Our results indicate that *rpoD* and 16S rRNA genes provide conflicting phylogenetic information concerning relatedness of *Azospirillum* species within clade L. This can be deduced from the low bootstrap support of branches in clade L when 16S rRNA data is included. Nevertheless, given that the rest of the tree branches showed improved confidence, sequencing and concatenation of *rpoD* with 16S rRNA and/or *nifH* would be recommended when possible for a more robust analysis. The inferred phylogenetic trees of individual or concatenated genes will be a valuable reference tool for an accurate typing of new azospirilla isolates.

To exemplify the use of *rpoD*-sequencing, we performed molecular typing on an historical collection of *Azospirillum* sp. strains maintained in the Plant Growth-Promoting Bacteria Laboratory of IMyZA-INTA, Argentine, since 1986. The analysis showed that the collection contains a considerable number of *A. brasilense* strains, members of *A. doebereinerae* species and also some strains closely related to *A. himalayense* and *A. soli* species. No isolates associated with clade L were detected, probably due to technical criteria during the selection (e.g. colony morphology). In addition, this approach allowed us to detect up to 7 putative undescribed species represented by strains that formed distant well-supported clades. When compared with their closest type strain, members of these clades shared lower identity values than the approximate species threshold that was estimated by calibration with genome similarity. Further DNA, biochemical and physiological analyses will be required to confirm if these groups are separate new species.

5. Conclusions

The use of 16S rRNA gene for genotyping of *Azospirillum* spp. presents limitations that may be associated to the great genome plasticity of these bacteria, specifically to HGT events. Genomic analysis allowed the detection of the *rpoD* gene as a suitable alternative genetic marker. A PCR-based method for *rpoD* genotyping was developed and used for obtaining an *Azospirillum* reference phylogenetic tree. With this method we were able to successfully genotype a collection of azospirilla and identify phylogenetic groups that may represent putative new species. Until whole-genome sequencing is broadly adopted as a standard method in bacterial identification, single-gene genotyping, such as the one presented here, outstands as relatively confident and low-cost techniques that allow quick identification of new bacterial isolates.

Conflict of interest

The authors have no conflicts of interest to declare.

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